0013-7227/94/1354-1401$03.00/0
Endocrinology

Vol. 135, No. 4
Printed in U.S.A.

Induction of Galanin Gene Expression in Gonadotropin-Releasing Hormone Neurons with Puberty in the Rat*

WINFRIED G. ROSSMANITH†, DANIEL L. MARKS, DONALD K. CLIFTON, AND ROBERT A. STEINER

Departments of Obstetrics and Gynecology (W.G.R., D.K.C., R.A.S.), Physiology and Biophysics (D.L.M., R.A.S.), and Zoology (R.A.S.) and the Population Center for Research in Reproduction (D.K.C., R.A.S.), University of Washington, Seattle, Washington 98195; and the Department of Obstetrics and Gynecology, University of Ulm (W.G.R.), D-89075 Ulm, Germany

ABSTRACT

The onset of puberty reflects the developmental activation of GnRH neurons whose secretory activity awakens the reproductive axis; however, the cellular mechanisms involved in this activational process remain poorly understood. GnRH neurons coexpress the neuropeptide galanin, and we have previously shown that galanin's level of coexpression is linked to the activity state of GnRH neurons. We theorized that altered expression of galanin by GnRH neurons may be an important mechanism related to activation of GnRH neurons at puberty. We examined two hypotheses related to this idea. First, we tested the hypothesis that expression of galanin messenger RNA (mRNA) in GnRH neurons is induced across the transition from prepubertal to adult life in the rat. To accomplish this, we used double label *in situ* hybridization and image analysis to compare cellular levels of galanin mRNA in GnRH neurons between groups of prepubertal and adult male and female rats. Levels of galanin mRNA within GnRH neurons increased significantly across puberty in both sexes. In females, galanin mRNA signal in GnRH neurons increased approximately 8-fold, whereas in males, cellular galanin mRNA signal levels increased about 2-fold. The number of identifiable GnRH neurons was not significantly different among the experimental groups. Next, we examined the hypothesis that pubertal induction of galanin mRNA in GnRH neurons reflects the activational effects of gonadal hormones associated with the onset of puberty. To test this, we killed groups of prepubertal male and female rats together with adult male and female animals that had been either castrated or sham castrated at a prepubertal age. In animals that had been prepubertally castrated, no developmental increase in galanin mRNA in GnRH neurons was observed, whereas in sham-castrated animals, levels of galanin mRNA in GnRH neurons were again shown to be higher in adult compared to prepubertal animals of both sexes, as had been demonstrated in the first experiment. We conclude that galanin message expression in GnRH neurons is induced during the transition from the juvenile to the adult state through a gonad-dependent process. This developmental increase in galanin gene expression is one mechanism by which the capacity for the synthesis and secretion of galanin by GnRH neurons may be enhanced, which, in turn, could facilitate the functional activity of GnRH neurons and amplify their trophic effect on the pituitary. (*Endocrinology* **135:** 1401–1408, 1994)

SEXUAL MATURATION in the rat reflects the onset of LH and FSH secretion and their stimulatory effect on gonadal activity. The induction of gonadotropin secretion at puberty is caused by a centrally mediated process that ultimately induces pulsatile GnRH release (see Ref. 1 for review). Although the cellular and molecular mechanisms underlying this developmental process are unknown, important clues to the identity of the controlling events are coming to light. One recent advance is the realization that the synthesis of GnRH is not, by itself, a rate-limiting factor governing the onset of puberty, as GnRH neurons have the capacity to synthesize and secrete GnRH long before the onset of puberty. Support for this precept derives from several lines of evidence. First, immunocytochemical studies have shown that the final distribution and peak number of GnRH neurons are established early in postnatal life (2–4). Second, *in situ* hybridization and Northern blot analyses have documented that cellular levels and total hypothalamic content of GnRH messenger RNA (mRNA) are achieved weeks before the onset of puberty (5, 6). Third, treatment of prepubertal animals with the neuroexcitatory amino acid *N*-methyl-D L-aspartate prematurely elicits GnRH secretion and advances the onset of puberty (7, 8). Together, these reports suggest that in the prepubertal state, the functional capacity of the GnRH neuron and, hence, the onset of puberty are not held in abeyance by limited expression of the GnRH gene. Rather, restraint of GnRH secretion during prepubertal life is attributable to some other gating phenomenon that is intrinsic to either the immature GnRH neuron or its afferent input.

We have focused our attention on genes that are coexpressed by GnRH neurons and have sought to link alterations in the expression of these candidate genes to the onset of puberty in the rat. One candidate is the neuropeptide galanin, which is synthesized and secreted by many GnRH neurons in the rat (9–11). Galanin's expression has been associated with alterations in the functional activity of the GnRH neuron. For example, during proestrus, when the enhanced secretory activity of GnRH neurons initiates the preovulatory LH surge (12–14), galanin mRNA levels in these neurons are concomitantly increased (15). In contrast, during lactation,

Received March 15, 1994.
Address all correspondence and requests for reprints to: Dr. Robert A. Steiner, Department of Obstetrics and Gynecology, RH-20, University of Washington, Seattle, Washington 98195.
* This work was supported by USPHS (NIH) Grants HD-12652, HD-12629, and HD-29039 and Deutsche Forschungsgemeinschaft Grant Ro 657/3-1.
† Permanent address: Department of Obstetrics-Gynecology, University of Ulm, Prittwitzstrasse 43, D-89075 Ulm, Germany.

when GnRH neurons are quiescent, galanin coexpression in GnRH neurons is virtually eliminated (16). Thus, in at least these two examples, the level of galanin expression in GnRH neurons is correlated with the activity state of these cells. Although the association between galanin expression and the activity state of GnRH neurons may be remarkable, its physiological significance remains uncertain.

Hypothalamic secretion of galanin is thought to play a role in the physiological control of gonadotropin secretion. When administered into the cerebral ventricles, galanin stimulates LH secretion (17). Conversely, the administration of either an antiserum to galanin or galantide, a galanin receptor antagonist, can block proestrous and steroid-induced LH surges (18, 19). These observations suggest that endogenous galanin serves a stimulatory function in the hypothalamic-pituitary axis and that enhanced expression of galanin by GnRH neurons acts in some manner to facilitate gonadotropin secretion. Based on these observations, we theorized that activation of GnRH neurons at puberty may be linked to the induction of galanin expression in these cells and, in turn, that this induction may be instrumental in controlling the onset of puberty. We tested two hypotheses related to the initial part of this theory. First, using double label *in situ* hybridization, we tested the hypothesis that cellular levels of galanin mRNA in GnRH neurons increase across pubertal development in male and female rats. Next, having observed that galanin message in GnRH neurons does, in fact, increase in both sexes over development, we examined the hypothesis that the increase in galanin expression in GnRH neurons is attributable to a gonad-independent mechanism; that is, it would occur in the absence of exposure to the pubertal rise in gonadal hormone production. We tested this hypothesis by examining the effect of prepubertal castration on the developmental induction of galanin message in GnRH neurons in male and female rats.

Materials and Methods

Animals

Adult (65-day-old) and juvenile (20-day-old) male and female Sprague-Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA). They were housed in pathogen-free, temperature- and light-controlled conditions (20 C; alternating light-dark cycle with 14 h of light and 10 h of darkness; lights on at 0700 h). The animals were given free access to tap water and rat chow. They were maintained in this environment for 5 days to control for stress, then killed at 70 days of age (adult) or 25 days of age (juvenile). (Puberty in Sprague-Dawley rats occurs around 35 days of age in females and between 40–60 days of age in males.)

Experimental design

Exp 1: galanin mRNA in GnRH neurons across pubertal development. In this experiment, we compared levels of galanin mRNA in GnRH mRNA neurons among groups of prepubertal (juveniles, 25 days of age) and adult (70 days of age) male and female rats. Each group of juvenile rats comprised five animals; each group of adult rats comprised six animals. The adult females were killed on diestrous day 1 of the estrous cycle; all animals were killed between 1000–1100 h by asphyxiation with CO_2 and then immediately decapitated. Trunk blood was collected and centrifuged, and the serum was stored at −20 C until assayed for sex steroid levels. The brains were rapidly removed, frozen on dry ice, and stored whole at −80 C.

Exp 2: role of gonadal hormones in pubertal induction of galanin mRNA in GnRH neurons. In this experiment, we tested whether prepubertal castration would prevent the induction of galanin message in GnRH neurons associated with the onset of puberty. To accomplish this, we compared levels of galanin mRNA in GnRH neurons in prepubertal male and female rats (25 days of age; n = 6 for each sex) with adults that had been either castrated or sham castrated at 25 days of age and allowed to mature to 70 days of age (n = 6 and 7, respectively, for both sexes). All surgical procedures were carried out while maintaining the animals under ether anesthesia. Animals were killed by rapid asphyxiation with CO_2, followed immediately by decapitation. Trunk blood was collected at the time of death. The brains were rapidly removed, frozen, and stored whole at −80 C until sectioning for the *in situ* hybridization procedure.

Tissue preparation

Immediately before sectioning, the brains were allowed to equilibrate in the cryostat chamber at −20 C. Coronal brain slices (20 μm) were cut with a cryostat, thaw-mounted onto silane-coated slides (Fischer Scientific, Fairlawn, NJ), and stored in air-tight boxes at −80 C until needed. Tissue was collected according to the rat atlas of Paxinos and Watson (20), beginning rostrally at the genu of the corpus calossum and continuing caudally 60 μm beyond the decussation of the anterior commissure.

Preparation of complementary RNA (cRNA) probes

^{35}S-Labeled galanin cRNA probe. The plasmid vector Bluescript containing a complementary DNA (cDNA) to rat galanin mRNA (21) was kindly provided by Dr. Maria Vrontakis (University of Manitoba, Winnipeg, Canada). The plasmid consisted of a 680-basepair segment of rat galanin cDNA inserted into the *Eco*RI site of Bluescript (Stratagene, La Jolla, CA). *Hin*dIII was used to linearize the cDNA, and the ^{35}S-labeled antisense riboprobe was generated through use of T7 RNA polymerase. The galanin probe transcription reactions had 25% of the total UTP as [^{35}S] UTP. The final double label hybridization solution contained a galanin cRNA probe concentration of 0.25 μg/ml·kilobase.

Digoxigenin-labeled GnRH cRNA probe. A 462-basepair digoxigenin-labeled cRNA probe to rat GnRH mRNA was used. The original plasmid containing the GnRH insert (22) was generously provided by Dr. A. Mason (Genentech, South San Francisco, CA). The probe was synthesized *in vitro* from linearized DNA with 400 μM digoxigenin-11-uridine-5′-triphosphate (Boehringer Mannheim, Indianapolis, IN), 100 μM unlabeled UTP, and 500 μM each of GTP, ATP, and CTP with SP6 RNA polymerase. Residual DNA was digested with deoxyribonuclease, and the cRNA probe was separated from unincorporated nucleotides on a Sephadex G-50 column (Boehringer Mannheim). The purified probe was diluted 1:50 in hybridization buffer for double label *in situ* hybridization. This concentration had been determined as optimal by a test *in situ* hybridization assay. Both probes were heat denatured before they were added to the final hybridization buffer.

The control experiments used to validate the integrity, binding kinetics, and specificity of both the digoxigenin-labeled GnRH and ^{35}S-labeled galanin probes were previously described (11, 22). The specificity of the cRNA probes was tested by application of sense probes, administration of excess unlabeled probes together with radioactively labeled and digoxigenin-labeled probes, and pretreatment of tissue with ribonuclease.

Double label in situ hybridization

The method used for double label *in situ* hybridization was previously described (11). In brief, sections were fixed in 4% paraformaldehyde and treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The slides were rinsed in 2 × SSC (1 × SSC = 150 mM NaCl and 15 mM Na citrate), dehydrated through a graded series of ethanol, delipidated in chloroform, rehydrated in a second ethanol series, and air dried. The tissue sections were prehybridized for 2 h at 60 C with hybridization buffer containing 2 mg/ml denatured total yeast RNA,

rinsed in 2 × SSC, dehydrated briefly in 70% ethanol, and air dried. The final hybridization buffer containing both probes was applied (60 μl/slide) to the tissue, covered with a Parafilm coverslip, and sealed with rubber cement. The slides were incubated in humid chambers overnight at 60 C. The next day, the tissue was treated with ribonuclease-A and washed under conditions of increasing stringency, including a wash at 65 C in 0.1 × SSC. After this, the slides were placed in 2 × SSC plus 0.05% Triton X-100 containing 2% normal sheep serum for 1 h. They were washed in buffer 1 (100 mM Tris-HCl, pH 7.5, and 150 mM NaCl) and incubated for 4 h at 37 C with antidigoxigenin antibody fragments conjugated to alkaline phosphatase (Boehringer Mannheim) diluted 1:1000 in buffer 1 containing 1% normal sheep serum and 0.3% Triton X-100. After washing, the slides were incubated in a chromagen solution for 6 h at 37 C. The reaction was stopped, and the slides were air dried and stored in the dark. After overnight drying, the slides were dipped in a 3% solution of parlodion (Fisher Scientific) in isoamyl acetate to prevent chemographic artifacts in the autoradiographic steps. The slides were air dried, dipped in Kodak NTB-2 photographic emulsion (Eastman Kodak, Rochester, NY), and then stored dried in light-tight boxes at 4 C. The slides were exposed for 10 days, then developed and coverslipped.

Semiquantitative analysis of cellular galanin mRNA levels in GnRH neurons

Slides were assigned a random three-letter code, alphabetized, and then read in random order with an automated image-processing system by an operator unaware of the animal's experimental group. We determined the number of silver grains per cell using a grain-counting program, as previously described (11). This system consisted of a PixelGrabber video acquisition board (Perceptics Corp., Knoxville, TN) attached to a Macintosh IIfx computer. Video images were obtained by a Dage model 65 camera (Dage-MTI, Michigan City, IN) attached to a Zeiss Axioskop (Zeiss, New York, NY). The microscope was equipped with a ×40 objective and a 100-watt mercury vapor epiillumination light source with a polarizing cube.

GnRH neurons were identified under brightfield illumination by the presence of purple staining in the cytoplasm of a cell, indicating the concentration of the digoxigenin-labeled cRNA probe for GnRH mRNA. Clusters of silver grains overlying these GnRH neurons, indicating the presence of the ^{35}S-labeled cRNA probe for galanin mRNA and its coexpression with GnRH mRNA, were counted under darkfield illumination by the computerized image processor. All identifiable GnRH neurons were analyzed for silver grain counts and included in the totals and group averages. This avoided the necessity of making the subjective decision of whether a particular GnRH cell was double labeled for galanin message. However, as some of the GnRH neurons may not, in fact, have expressed galanin message above detectable limits, the grain count summaries may underestimate actual galanin mRNA levels in an undefined subset of galanin-expressing GnRH neurons. The impact of this method of analysis is to minimize differences among experimental groups and to give a conservative estimate of real changes taking place in galanin mRNA signal levels. Twenty-one sections per brain were analyzed for the number of grains per cell. Sections were equally spaced throughout the diagonal band of Broca (DBB) and preoptic area (POA), with the most caudal section at the level of the suprachiasmatic nucleus and the rostral aspect of the lateral hypothalamus. The number of grains per cell is referred to as the galanin mRNA signal level.

Sex steroid assays

Serum levels of testosterone in the male and estradiol in the female animals were measured by RIAs. Testosterone was extracted from plasma samples with anhydrous ether and then measured by RIA after TLC (23). Estradiol was measured by RIA after chromatography of the plasma samples (24). All samples were measured in a single assay. The intraassay coefficients of variation were 10% and 7% for the testosterone and estradiol assays, respectively.

Statistical analysis

For all experiments, n refers to the number of experimental animals within a group, and this was the n used in the analysis. For cellular GnRH mRNA or galanin mRNA content determinations, the mean grains per cell from individual animals were used to calculate the mean ± SEM for each group. An average of 24 GnRH neurons in each animal were examined for calculation of the mean grains per cell. The differences between groups were assessed by analysis of variance (ANOVA) or Student's t test for unpaired observations when appropriate. When the ANOVA indicated a significant difference between groups within an experiment, Fisher's protected least significant difference test was used to identify significant differences between groups. The rejection level for statistical tests was set at $\alpha = 0.05$.

Results

GnRH neurons expressing galanin mRNA were observed in the anterior hypothalamic area (AHA), horizontal and vertical limbs of the DBB (DBBH and DBBV, respectively), the medial POA (MPOA), the median POA, the lateral POA, organum vasculosum of the lamina terminalis, and the medial septum of all animals. Simultaneous bright- and darkfield images of representative cells from the experiments are shown in Fig. 1.

Exp 1: galanin mRNA in GnRH neurons across pubertal development

Levels of galanin expression in GnRH neurons varied as a function of age and sex. Analysis by two-way ANOVA (age *vs.* sex) indicated that levels of galanin mRNA in GnRH neurons increased significantly across development in both sexes ($P < 0.0001$). Furthermore, levels of galanin message in GnRH neurons and their developmental patterns were sexually differentiated ($P < 0.005$). Because the overall analysis demonstrated that there were significant effects of age and sex on galanin message levels, additional statistical analyses were performed.

Galanin mRNA in GnRH neurons of males across puberty

In male rats, levels of galanin mRNA in GnRH neurons were significantly higher in adult compared with prepubertal animals (juveniles, 7 ± 1; adults, 17 ± 3 grains/cell; $P < 0.004$; Fig. 2). GnRH cells located in the MPOA and caudal DBBH showed the largest increase in galanin mRNA content in GnRH neurons across puberty (data not shown). The number of GnRH neurons found in the brain sections examined was not significantly different between age groups (juveniles, 22 ± 3; adults, 24 ± 1 cells).

Galanin mRNA in GnRH neurons of females across puberty

In female rats, levels of galanin mRNA in GnRH neurons were significantly higher in adult compared with prepubertal animals (juveniles, 6 ± 3; adults, 47 ± 9 grains/cell; $P < 0.001$; Fig. 2). The increase in galanin message levels occurred primarily in GnRH cells of the caudal DBBH and MPOA, whereas galanin mRNA levels in GnRH neurons located in more rostral areas (rostral DBBV) were not statistically different between age groups (data not shown). The number of

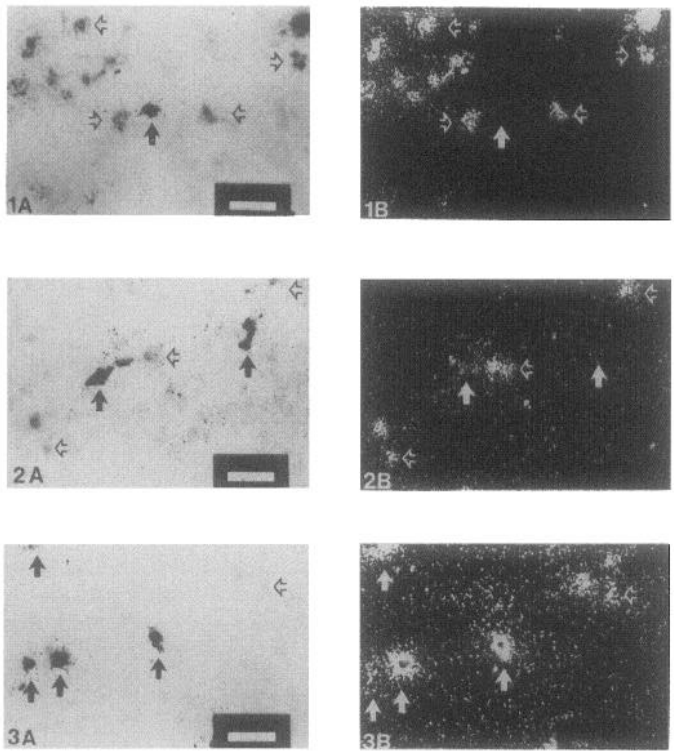

FIG. 1. Photomicrograph of neurons in the vertical limb of the DBB under brightfield (*left*) and darkfield (*right*) illumination after double label *in situ* hybridization for GnRH mRNA and galanin mRNA. Displayed are sections from a juvenile female (1A and 1B), a prepubertally castrated adult female (2A and 2B), and a prepubertally sham-castrated adult female rat (3A and 3B). Under brightfield illumination, the presence of digoxigenin-labeled GnRH mRNA is indicated by dark-stained cell bodies. Silver grains from the radiolabeled galanin cRNA probe appear as *black dots* in brightfield and *white dots* in darkfield. Clusters of silver grains mark cells containing galanin mRNA (*open arrows*). Identical cells identified as expressing GnRH mRNA (brightfield) and galanin mRNA (darkfield) are marked by *solid white and black arrows*.

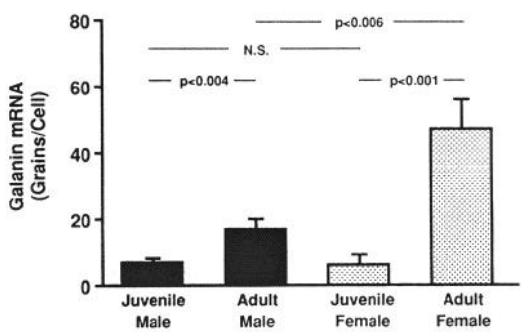

FIG. 2. The effects of age and gender on galanin mRNA signal in GnRH neurons throughout the rostral hypothalamus of the rat. *Bars* represent mean signal levels (grains per cell), and *error lines* indicate the SEM. N.S. indicates there was no significant difference between the two groups. Each group of juvenile rats comprised five animals; each group of adult rats comprised six animals.

GnRH neurons was not significantly different between age groups (juveniles, 16 ± 5; adults, 15 ± 4 cells).

Galanin mRNA in GnRH neurons in prepubertal animals: males vs. females

The signal for galanin mRNA in GnRH neurons was just above the limit of detectability before puberty (25 days of age) and not sexually differentiated at this developmental stage (prepubertal males, 9 ± 2; prepubertal females, 9 ± 3 grains/cell; Fig. 2). In addition, there were no differences between groups in any of the individual anatomical areas examined (data not shown). The number of GnRH neurons was not significantly different between groups (prepubertal males, 30 ± 4; prepubertal females, 29 ± 4 cells).

Galanin mRNA in GnRH neurons in adult animals: males vs. females

Levels of galanin mRNA in GnRH neurons were sexually differentiated in adult rats (Fig. 2), with galanin message levels in GnRH neurons being approximately 280% higher in females compared to males ($P < 0.006$). GnRH cells located in the MPOA and caudal DBBH showed the largest difference in galanin mRNA content in GnRH neurons between groups (data not shown).

Exp 2: effect of prepubertal castration on the developmental rise of galanin expression in GnRH neurons

Males. The pubertal increase in levels of galanin mRNA in GnRH neurons was absent in adult males that had been castrated before the onset of puberty. In this case, the cellular galanin mRNA content in GnRH neurons of adults that had been castrated prepubertally was indistinguishable from that of juvenile animals (juvenile males, 2 ± 1; prepubertally castrated adult males, 3 ± 1 grains/cell). In control animals that had been sham castrated before puberty, levels of galanin mRNA in GnRH neurons increased across development (juvenile males, 2 ± 1; prepubertally sham-castrated adult males, 13 ± 4 grains/cell; $P < 0.01$; Fig. 3), confirming the observations made in Exp 1. The number of GnRH neurons was not significantly different among the age groups (juveniles, 30 ± 3; prepubertally castrated adults, 27 ± 5; prepubertally sham-castrated adults, 22 ± 4 grains/cell).

Females. The pubertal increase in galanin mRNA content in GnRH neurons was absent in adult females that had been ovariectomized before the onset of puberty (juvenile females, 2 ± 1; prepubertally castrated adult females, 4 ± 2 grains/cell; $P > 0.4$). In control animals that had been sham castrated before puberty, levels of galanin mRNA in GnRH neurons increased significantly across age, consistent with the results of the first experiment (juveniles, 2 ± 1; prepubertally sham-castrated adults, 31 ± 10 grains/cell; $P < 0.01$; Fig. 3). Again, as in Exp 1, the increase in galanin message content was prominent in GnRH cells of the caudal DBBH and MPOA (data not shown). The number of GnRH neurons was not significantly different among the experimental groups (ju-

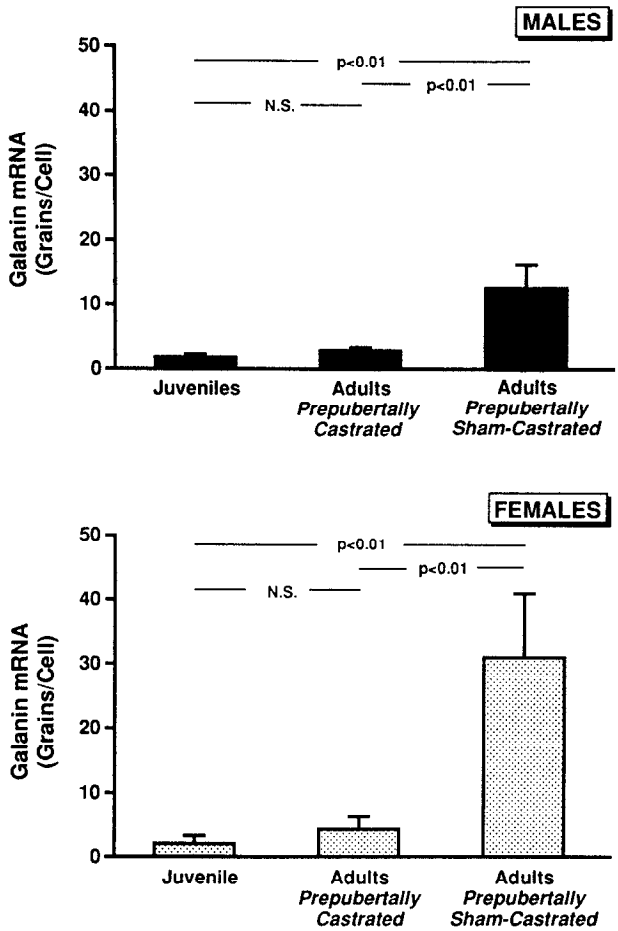

FIG. 3. The effects of prepubertal castration on galanin mRNA signal in GnRH neurons throughout the rostral hypothalamus in male (*upper panel*) and female (*lower panel*) rats. *Bars* represent mean signal levels (grains per cell), and *error lines* indicate the SEM. N.S. indicates there was no significant difference between the two groups. Each group of juvenile rats comprised six animals; adult male and adult female groups comprised six and seven animals, respectively.

venile, 22 ± 4; prepubertally sham-castrated adult, 25 ± 5; prepubertally castrated adult, 22 ± 4 cells).

Expression of galanin mRNA in non-GnRH neurons

The overall distribution and cellular levels of galanin mRNA in non-GnRH neurons were not different among the experimental groups (males: juveniles, 161 ± 5; prepubertally castrated adults, 150 ± 8; prepubertally sham-castrated adults, 145 ± 19 grains/cell; females: juveniles, 175 ± 12; prepubertally castrated adults, 169 ± 13; prepubertally sham-castrated adults, 155 ± 14 grains/cell; data not shown).

Serum levels of testosterone in males and estradiol in females

Serum levels of testosterone and estradiol for each experimental group are displayed in Table 1. As expected, in males, serum levels of testosterone were significantly higher in adult compared with prepubertal animals and were profoundly reduced after castration. Likewise in females, serum levels of estradiol were significantly higher in adult compared with prepubertal animals and were reduced after ovariectomy. These observations support the physiological assumptions about the developmental state of the animals and confirm the effectiveness of the surgical castrations.

TABLE 1. Serum levels of testosterone and estradiol in the experimental groups

	Testosterone (ng/ml)	Estradiol (pg/ml)
Exp 1		
Juvenile males (n = 5)	0.5 ± 0.1	
Adult males (n = 6)	8 ± 6	
Juvenile females (n = 5)		4 ± 1
Adult females (n = 6)		25 ± 5
Exp 2		
Juvenile males (n = 6)	0.5 ± 0.2	
Prepubertally castrated adult males (n = 7)	0.3 ± 0.1	
Prepubertally sham-castrated adult males (n = 6)	11 ± 1	
Juvenile females (n = 6)		4 ± 1
Prepubertally castrated adult females (n = 7)		1 ± 1
Prepubertally sham-castrated adult females (n = 6)		21 ± 7

Values are expressed as the mean ± SEM. Details on treatment regimens can be found under the *Experimental design* section in *Materials and Methods*.

Discussion

We report that in rats, levels of galanin mRNA in GnRH neurons increase over development in both sexes and that the pattern of this induction phenomenon is sexually differentiated. These results help to clarify and extend the earlier observations of Gabriel *et al.* (25), who showed that galanin peptide levels in the median eminence increase in association with pubertal maturation and that this increase is greater in females than males. These observations suggest that induction of galanin message and protein synthesis in GnRH neurons at puberty accounts at least in part for the developmental increase in total hypothalamic galanin content. Galanin in the median eminence originates from a variety of sources, including GH-releasing hormone (GHRH) neurons (26), and we have previously shown that GHRH neurons, like GnRH neurons, show a marked increase in galanin mRNA content over the course of pubertal development (27). We conclude that the increase in galanin content in the median eminence that occurs over puberty reflects increased galanin synthesis in hypothalamic neurons, including those containing GnRH and GHRH. Taken together, these observations establish that the onset of puberty occurs in association with changes in galanin gene expression within neurons of the reproductive neuroendocrine system as well as the GH axis, and we suggest that galanin may serve a common function in these interrelated developmental processes.

During prepubertal life, the reproductive axis is suspended in a state of relative quiescence, then awakened by the initiation of pulsatile GnRH secretion, which ultimately drives the onset of puberty (1). Despite their apparent prepubertal somnolence, GnRH neurons have the capacity to

synthesize and secrete GnRH well before the onset of puberty (3, 5, 28). Cellular levels of GnRH message remain relatively constant across the transition from early juvenile life to adulthood (6, 22), suggesting that little or no change occurs in biosynthetic demand for GnRH as a function of pubertal maturation. Pharmacological challenges with NMDA receptor analogues are capable of provoking GnRH release in prepubertal rats, testifying to the functional integrity of the GnRH secretory pathway (7, 8). Hence, the capacity to synthesize and secrete GnRH is in place well before the onset of puberty (29, 30). However, it would appear that GnRH neurons in the prepubertal animal either release insufficient quantities of GnRH or secrete GnRH in a manner that is incapable of stimulating gonadotropin secretion. Based on the foregoing observations, we favor the hypothesis that in prepubertal animals, GnRH neurons do, in fact, secrete GnRH, but do so in a nonpulsatile or dysynchronous fashion, which is ineffective in stimulating gonadotropin secretion.

A shift in the pattern of GnRH secretion at puberty may be driven by alterations in the activity of excitatory or inhibitory inputs to the GnRH system (22, 31). Alternatively, alterations intrinsic to the GnRH neuron, such as the production of cotransmitters and their receptors, may provide a mechanism for modification of GnRH secretion that is independent of or complimentary to changes in afferent activity. Is it possible that increased galanin expression in GnRH neurons at puberty either enables GnRH neurons to generate discrete pulses or synchronizes pulsatile discharge among individual GnRH neurons? This would transform GnRH secretion from an ineffective continuous release mode to a pulsatile pattern that greatly enhances gonadotropin secretion (32, 33). If this were the case, how might it be accomplished? Galanin is cosecreted from cholinergic neurons projecting from the septum to the hippocampus and is thought to act presynaptically to modulate acetylcholine secretion during and subsequent to a secretory burst (34, 35). By analogy, galanin cosecreted with GnRH could act in an autocrine fashion to modulate the secretion of GnRH. Accordingly, after the release of vesicles containing both GnRH and galanin at the median eminence, GnRH itself would travel via the portal circulation to stimulate the synthesis and release of gonadotropins, whereas galanin would act presynaptically to facilitate further GnRH release during a secretory bout (19). In the absence of galanin, GnRH would spill continuously in an unregulated nonpulsatile fashion into the portal vasculature. Under these circumstances, only minimal stimulation of gonadotropin secretion would occur. These conditions would prevail, for example, in the prepubertal state and during lactation, when there is little demand for gonadotropin action. With the onset of puberty, the enhancement of galanin secretion may act as an autocrine cotransmitter with GnRH to provide an ultrashort loop feedback signal to the GnRH neurons whose function it is to remodel the pattern of GnRH secretion from that of the prepubertal to that of the adult animal. This model predicts that coexpression of galanin in GnRH neurons facilitates the organization of GnRH release into distinct pulses, thereby providing an effective stimulus to the pituitary gonadotrope (36, 37). This report together with the earlier observations that galanin is coexpressed with GnRH in the rat brain (9–11), that exogenously administered galanin stimulates LH secretion (17), that galanin-binding sites are located in the immediate vicinity of GnRH nerve terminals in the median eminence (38), and that pharmacological blockade of galanin receptors in the hypothalamus inhibits LH secretion (19) are at least consonant with this model for the functional significance of galanin in GnRH neurons.

Our observation that the increased galanin mRNA content of adult GnRH neurons is prevented by prepubertal castration suggests that the pubertal induction of galanin expression is gonad dependent and underscores the importance of gonadal hormones in achieving sexual maturation of the rodent brain. These results are in full accord with those of Merchenthaler *et al.* (39), which showed that the number of LHRH-like galanin immunoreactive neurons found in adult animals can be manipulated by neonatal castration. Although the gonadal factors that stimulate galanin expression in GnRH neurons at puberty remain to be identified, plausible candidates would include the sex steroids. In the female rat, levels of galanin mRNA in hypothalamic fragments (40) and GnRH neurons are correlated with circulating levels of estradiol. When plasma estradiol concentrations are low, such as during lactation and after castration, galanin message levels in GnRH neurons are concomitantly low (16, 37, 41), and the postcastration decline in the galanin mRNA content of GnRH neurons can be reversed by replacement with physiological doses of estradiol (15). Furthermore, the galanin mRNA content in GnRH neurons is highest during the proestrous LH surge, when plasma estradiol levels are at their highest physiological level (15). Therefore, it seems plausible that rising plasma titers of estradiol associated with pubertal development in the female induce the expression of galanin in GnRH neurons as the animal enters adulthood. The gonadal factors responsible for producing elevated levels of galanin gene expression in GnRH neurons of the adult male rat could be either testosterone or estrogen, presumably derived through aromatization. Testosterone is a possible candidate for mediating this effect, particularly in view of the fact that testosterone can induce galanin expression in vasopressinergic cells coexpressing galanin in the bed nucleus of the stria terminalis (42). Regardless of the identity of the factor responsible for inducing galanin mRNA in GnRH neurons of the adult male rat, the pubertal induction of galanin message in GnRH neurons is gonad dependent. Finally, whether analogous reasoning applies to the brain of primate species remains to be determined, as the mechanisms controlling the onset of puberty in the primate differ markedly from those in the rat (43).

The pubertal increase in galanin expression in GnRH neurons is nearly 4-fold greater in females than males, resulting in the sexual differentiation of this phenomenon in adult animals. Earlier studies demonstrated that GnRH neurons in the adult female contain higher levels of galanin peptide and message than GnRH neurons in males (11, 44). The results we present here confirm these earlier observations and demonstrate that these differences develop over

puberty and depend on the presence of the gonads. The fact that levels of galanin mRNA in GnRH neurons are higher in the adult female than in the male may relate to some unique function for galanin in GnRH neurons in the female. The ability to generate a LH surge in response to estrogen is sexually differentiated in the rat, with only females having this capacity (45), and galanin expression in GnRH neurons has been linked to this process (18, 19). Thus, the mechanism for generation of the preovulatory GnRH surge in the female may be rooted in the ability of estrogen to induce the expression of galanin mRNA in GnRH neurons.

What happens to galanin message levels in non-GnRH neurons over development? As there are thousands of galanin cells widely dispersed through the hypothalamus, we attempted to address this question by measuring galanin mRNA in a randomly selected population. Galanin neurons represent a highly heterogenous population from which it is impossible to obtain a truly representative sample. Notwithstanding, the results we obtained suggest that increasing galanin gene expression over development is not characteristic of all galanin neurons, but is, instead, a phenomenon restricted to certain subpopulations. As already noted, galanin message levels also increase with development in GHRH neurons (27). It may be coincidental, but is certainly striking, that the ability of the neuroendocrine axis to generate pulses of both GH or LH occurs with the onset of puberty. This event is associated with a profound induction of galanin gene expression in GnRH and GHRH neurons, respectively.

It is conceivable that galanin's action is at the level of the pituitary and that at puberty, galanin stimulates gonadotropin secretion or facilitates the action of GnRH directly at the gonadotrope. Galanin is, in fact, capable of stimulating LH secretion from isolated pituitary cells (46) and can elicit LH secretion after administration in the cerebral ventricles (17). However, several other lines of evidence would argue against a pituitary site of action for galanin released at GnRH nerve terminals. First, galanin is produced in the pituitary (47) and presumably plays some paracrine function there (48). This fact makes it difficult to understand how relatively low concentrations of blood-borne galanin transported in the portal circulation from the brain could serve as an effective messenger to another gland producing that same substance in abundance. Second, in addition to its coexpression by GnRH neurons, galanin is coexpressed with GHRH in hypothalamic neurons, whose terminals also project to the median eminence (26, 27). As at least two and possibly more unrelated populations of hypophysiotropic neurons cosecrete galanin from their terminals in the median eminence, it seems implausible that target cells for both would be in the pituitary. These arguments do not disprove a pituitary site of action for galanin; however, based on our current knowledge, this hypothesis seems less attractive.

In summary, we have shown that in rats, the expression of galanin mRNA in GnRH neurons is low before puberty and increases over development in both sexes. This rise in galanin message levels is greater in females than males and is dependent on the presence of the gonads. Although the physiological relevance of enhanced galanin expression in GnRH neurons at puberty remains to be determined, galanin's possible role in facilitating the development of pulsatile secretion of GnRH and the induction of gonadotropin secretion at puberty deserves thoughtful and critical analysis.

Acknowledgments

The authors extend their appreciation to Emilia Kabigting and Beth Tiemens for their excellent technical assistance, to Dr. David Hess at the Oregon Primate Research Center for performing the testosterone and estradiol assays, and to Terre Poppe for her expert editorial and secretarial help.

References

1. **Ojeda SR, Urbanski HF** 1988 Puberty in the rat. In: Knobil E, Neill J (eds) The Physiology of Reproduction. Raven Press, New York, pp 1699–1737
2. **Wray S, Hoffman G** 1986 Postnatal morphological changes in rat LHRH neurons correlated with sexual maturation. Neuroendocrinology 43:93–97
3. **Silverman AJ, Livine I, Witkin WJ** 1994 The gonadotropin-releasing hormone (GnRH) neuronal systems: immunocytochemistry and *in situ* hybridization. In: Knobil E, Neill J (eds) The Physiology of Reproduction. Raven Press, New York, vol 2:1683–1709
4. **Schwanzel-Fukuda M, Pfaff DW** 1989 Origin of luteinizing hormone releasing hormone neurons. Nature 338:161–164
5. **Wiemann JN, Clifton DK, Steiner RA** 1989 Pubertal changes in gonadotropin-releasing hormone and proopiomelanocortin gene expression in the brain of the male rat. Endocrinology 124:1760–1767
6. **Jakubowski M, Blum M, Roberts JL** 1991 Postnatal development of gonadotropin-releasing hormone and cyclophilin gene expression in the female and male rat brain. Endocrinology 128:2702–2708
7. **Urbanski HF, Ojeda SR** 1987 Activation of luteinizing hormone-releasing hormone release advances the onset of female puberty. Neuroendocrinology 46:273–276
8. **Urbanski HF, Ojeda SR** 1990 A role for *N*-methyl-D-aspartate (NMDA) receptors in the control of LH secretion and initiation of female puberty. Endocrinology 126:1774–1776
9. **Coen CW, Montagnese C, Opacka-Juffry J** 1990 Coexistence of gonadotrophin-releasing hormone and galanin: immunohistochemical and functional studies. J Neuroendocrinol 2:107–111
10. **Merchenthaler I, López FJ, Negro-Vilar A** 1990 Colocalization of galanin and luteinizing hormone-releasing hormone in a subset of preoptic hypothalamic neurons: anatomical and functional correlates. Proc Natl Acad Sci USA 87:6326–6330
11. **Marks DL, Wiemann JN, Burton KA, Lent KL, Clifton DK, Steiner RA** 1992 Simultaneous visualization of two cellular mRNA species by use of a new double *in situ* hybridization method. Mol Cell Neurosci 3:395–405
12. **Sarkar DK, Chiappa SA, Fink G** 1976 Gonadotropin-releasing hormone surge in proestrous rats. Nature 264:461–463
13. **Leadem CA, Kalra SP** 1984 Stimulation with estrogen and progesterone of luteinizing hormone (LH)-releasing hormone release from perifused adult female rat hypothalami: correlation with the LH surge. Endocrinology 114:51–56
14. **Lee WS, Smith MS, Hoffman GE** 1990 Luteinizing hormone-releasing hormone neurons express *fos* protein during the proestrous surge of luteinizing hormone. Proc Natl Acad Sci USA 87:5163–5167
15. **Marks DL, Smith MS, Vrontakis M, Clifton DK, Steiner RA** 1993 Regulation of galanin gene expression in gonadotropin-releasing hormone neurons during the estrous cycle of the rat. Endocrinology 132:1836–1844
16. **Marks DL, Smith MS, Clifton DK, Steiner RA** 1993 Regulation of GnRH and galanin gene expression in GnRH neurons during lactation in the rat. Endocrinology 133:1450–1458

17. **Sahu A, Crowley WR, Tatemoto K, Balasubramaniam A, Kalra SP** 1987 Effects of neuropeptide Y, NPY analog (norleucine-NPY), galanin and neuropeptide K on LH release in ovariectomized (OVX) and OVX estrogen, progesterone treated rats. Peptides 8:921–926
18. **López FJ, Meade EH, Negro-Vilar A** 1993 Endogenous galanin modulates the gonadotropin and prolactin proestrous surges in the rat. Endocrinology 132:795–800
19. **Sahu A, Xu B, Kalra SP** 1994 Role of galanin in stimulation of pituitary luteinizing hormone secretion as revealed by a specific receptor antagonist, galantide. Endocrinology 134:529–536
20. **Paxinos G, Watson C** 1986 The Rat Brain in Stereotaxic Coordinates. Academic Press, New York
21. **Vrontakis ME, Peden LM, Duckworth ML, Friesen HG** 1987 Isolation and characterization of a complimentary DNA (galanin) clone from estrogen-induced pituitary tumor messenger RNA. J Biol Chem 262:16755–16758
22. **Wiemann JN, Clifton DK, Steiner RA** 1990 Gonadotropin-releasing hormone messenger ribonucleic acid levels are unaltered with changes in the gonadal hormone milieu of the adult male rat. Endocrinology 127:523–532
23. **Resko JA, Malley A, Begley D, Hess DL** 1973 Radioimmunoassay of testosterone during fetal development of the rhesus monkey. Endocrinology 93:156–161
24. **Goodman RL** 1978 A quantitative analysis of the physiological role of estradiol and progesterone in the control of tonic and surge secretion of luteinizing hormone in the rat. Endocrinology 102: 142–150
25. **Gabriel SM, Kaplan LM, Martin JB, Koenig JI** 1989 Tissue-specific sex differences in galanin-like immunoreactivity and galanin mRNA during development in the rat. Peptides 10:369–374
26. **Meister B, Scanlon MF, Hokfelt T** 1990 Occurrence of galanin-like immunoreactivity in growth hormone-releasing factor (GRF)-containing neurons of the monkey (*Macaca fascicularis*) infundibular nucleus and median eminence. Neurosci Lett 119:136–139
27. **Delemarre-van de Waal HA, Burton KA, Kabigting EB, Steiner RA, Clifton DK** 1994 Expression and sexual dimorphism of galanin messenger RNA in growth hormone releasing factor neurons of the rat during development. Endocrinology 134:665–671
28. **Cameron JL, McNeill TH, Fraser HM, Bremner WJ, Clifton DK, Steiner RA** 1985 The role of endogenous gonadotropin-releasing hormone in the control of luteinizing hormone and testosterone secretion in the juvenile male monkey, *Macaca fascicularis*. Biol Reprod 33:147–156
29. **Bourguignon JP, Franchimont P** 1984 Puberty-related increase in episodic LHRH release from rat hypothalamus *in vitro*. Endocrinology 114:1941–1943
30. **Matsumoto AM, Karpas AE, Southworth MB, Dorsa DM, Bremner WJ** 1986 Evidence for activation of the central nervous system-pituitary mechanism for gonadotrophin secretion at the time of puberty in the male rat. Endocrinology 119:362–369
31. **Adams LA, Steiner RA** 1988 Puberty. Oxf Rev Reprod Biol 10:1–52
32. **Belchetz PE, Plant TM, Nakai Y, Knobil E** 1978 Hypophysial responses to continuous and intermittent delivery of hypothalamic gonadotropin-releasing hormone. Science 202:631–633
33. **Southworth MB, Matsumoto AM, Gross KM, Soules MR, Bremner WJ** 1991 The importance of signal pattern in the transmitssion of endocrine information: pitutiary gonadotropin responses to continuous and pulsatilie gonadotropin-releasing hormone. J Clin Endocrinol Metab 72:1286–1289
34. **Fisone G, Wu CF, Consolo S, Nordstrom O, Brynne N, Bartfai T, Melander T, Hokfelt T** 1987 Galanin inhibits acetylcholine release in the ventral hippocampus of the rat: histochemical, autoradiographic, *in vivo*, and *in vitro* studies. Proc Natl Acad Sci USA 84:7339–7343
35. **Consolo S, Palazzi E, Bertorelli R, Fisone G, Crawley J, Hokfelt T, Bartfai T** 1990 Functional aspects of acetylcholine-galanin coexistence in the brain. Prog Brain Res 84:279–287
36. **Clayton RN, Channabasavaiah K, Stewart JM, Catt KJ** 1982 Hypothalamic regulation of pituitary gonadotropin-releasing hormone receptors: effects of hypothalamic lesions and gonadotropin releasing hormone antagonist. Endocrinology 110:1108–1115
37. **Marks DL, Clifton DK, Steiner RA**, Induction of galanin gene expression in GnRH neurons with puberty in the rat. Lawson Wilkins Pediatric Endocrine Society and European Society for Pediatric Endocrinology, Fourth Joint Meeting, San Francisco CA, 1993 (Abstract 173)
38. **Lagny-Pourmir I, Epelbaum J** 1992 Regional stimulatory and inhibitory effects of guanine nucleotides on [^{125}I]galanin binding in rat brain: relationship with the rate of occupancy of galanin receptors by endogenous galanin. Neuroscience 49:829–847
39. **Merchenthaler I, Lennard DE, López FJ, Negro-Vilar A** 1993 Neonatal imprinting predetermines the sexually dimorphic, estrogen-dependent expression of galaini in luteinizing hormone-releasing hormone neurons. Proc Natl Acad Sci USA 90:10479–10483
40. **Gabriel SM, Washton DL, Roncancio JR** 1992 Modulation of hypothalamic galanin gene expression by estrogen in peripubertal rats. Peptides 13:801–806
41. **Marks DL, Lent KL, Rossmanith WG, Clifton DK, Steiner RA** 1994 Activation-dependent induction of galanin mRNA expression in GnRH neurons in the rat. Endocrinology 134:1991–1998
42. **Miller MA, Kolb PE, Raskind MA** 1993 Testosterone regulates galanin gene expression in the bed nucleus of the stria terminalis. Brain Res 611:338–341
43. **Plant TM** 1994 Puberty in primates. In: Knobil E, Neill J (eds) The Physiology of Reproduction. Raven Press, New York, vol 2: 453–485
44. **Merchenthaler I, López FJ, Lennard DE, Negro-Vilar A** 1991 Sexual differences in the distribution of neurons coexpressing galanin and luteinizing hormone-releasing hormone in the rat brain. Endocrinology 129:1977–1986
45. **Neill JD** 1972 Sexual differences in the hypothalamic regulation of prolactin secretion. Endocrinology 90:1154–1160
46. **López FJ, Merchenthaler I, Ching M, Wisniewski MG, Negro-Vilar A** 1991 Galanin: a hypothalamic-hypophysiotropic hormone modulating reproductive functions. Proc Natl Acad Sci USA 88:4508–4512
47. **Kaplan LM, Gabriel SM, Koenig JI, Sunday ME, Spindel ER, Martin JB, Chin WW** 1988 Galanin is an estrogen-inducible, secretory product of the anterior pituitary. Proc Natl Acad Sci USA 85:17408–17412
48. **Merchenthaler I, López FJ, Negro-Vilar A** 1993 Anatomy and phyusiology of central galanin-containing pathways. Prog Neurobiol 40:711–769